A 0.123% Stevia/Aspartame 91/9 Aqueous Solution Balances the Effects of the Two Substances, and may thus be a Safer and Tastier Sweetener to be Used

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Abstract: Sweeteners are presently largely consumed all over the world, essentially aspartame (North America, Europe) and stevia (South America, Asia). Aspartame has a pleasant taste but present some adverse effects; stevia has very few adverse effects but has not the sweetest taste. Using ants as biological models, we here examined if a 0.123% solution of stevia/aspartame 91/9 might have both a pleasant taste and nearly no adverse effects. We found that it did not change the ants' food consumption while aspartame increased it and stevia slightly decreased it. It did not affect their locomotion, precision of reaction and response to pheromones as aspartame did. It did not increase their audacity as aspartame largely and stevia somewhat did. It did not affect the ants' brood caring behavior and cognition as aspartame did, and it did not impact the conditioning ability and memory as aspartame drastically and stevia slightly did. Confronted to sugar water and a stevia/aspartame 91/9 solution, the ants equally drunk the two solutions, while having the choice between aspartame and sugar, they soon nearly exclusively chose the sugar, and while in presence of stevia and sugar, the ants progressively chose the sugar. Very probably aspartame enhanced the taste of stevia, and as the latter contains a true glycoside, a stevia/aspartame 91/9 solution did not affect the ants' physiology and ethology as pure aspartame did. In front of sugar and a stevia/aspartame ca 96/4 solution, the ants chose the sugar. Thus, a 0.123% solution in which 9% aspartame (and no less) is mixed to 91% stevia (and no more) appears to constitute a safe and tasty sweetener which could be used instead of solutions containing only aspartame or stevia.

Keywords: Ant, cognition, food additive, locomotion, memory.

INTRODUCTION

Sweeteners are actually more and more consumed all over the world. The two more used sweeteners are aspartame (in North America and Europe) and 'stevia' (in South America and Asia). Aspartame is not a glycoside (Figure **1**) but has a strong sweetened taste that leads to physiological and ethological perturbations such as enhancing the search for food, increasing food consumption, decreasing several cognitive abilities and the precision of reaction. Moreover it soon hydrolyzes into dangerous substances such as phenylalanine, methanol and finally formaldehyde [1-3]. Stevia is a true glycoside extracted from the plant *Stevia rebaudiana* (Figure **1**), having so very few adverse physiological and ethological effects. However, it has not a nice sweetened taste; it slightly leads to less food consumption, and finally gives rise to steviol which is not without harmful effects [4, 5]. Each of these two sweeteners should be consumed in small amount, this concerning above all aspartame. We already examined the effects of these two sweeteners using ants as biological models (aspartame [6], stevia [paper accepted for publication]). On basis of these experimentations, we realized that the two sweeteners have somewhat opposite ethological effects and properties. We then thought that an adequate mixture of the two sweeteners evidently contains less amount of each sweetener but also might possess the nice taste of aspartame, be rather safe since containing a true glycoside, and cancel the unwanted effects of each two sweeteners. We thus aimed to use once more ants as biological models for examining physiological and ethological effects of a stevia/aspartame mixture in aqueous solution (the controls being the effects under a sugar diet), and this without any conflict of interest.

Why Using Ants as Biological Models?

Most biological processes are quite similar for all animals, including humans (i.e. genetics, metabolism, nervous cells functioning). Consequently, a lot of invertebrates and vertebrates are used as models for studying biological subjects [7-9]. Invertebrates are

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more and more used for this goal because they offer scientists many advantages, among others a short life cycle, a simple anatomy, and being available in large numbers [10, 11]. Some species are largely used as biological models, for instance, the flatworm *Dendrocelium lacteum*, the nematode worm *Caenorhabdotes elegans*, the mollusk *Aplysia californica*, the beetle *Tribolium castaneum*, the fruit fly *Drosophila melanogaster*, and the domestic bee *Apis mellifera*. Among the invertebrates, insects, especially social hymenoptera and among them, bees, are advantageously used as biological models [12, 13], but ants too can be used. Indeed, colonies containing thousands of ants can easily be maintained in laboratories, at low cost and very conveniently, throughout the entire year. Ants are among the most complex and social invertebrate animals as for their morphology, physiology, social organization and behavior. They are among the most morphologically evolved hymenoptera, having indeed a unique resting position of their labium, mandibles and maxilla [14], as well as a lot of glands emitting numerous efficient pheromones [15]. Their societies are highly organized with a strong division of labor, an age-based polyethism and a social regulation [16]. Their behavior is well developed: they care for their brood, build sophisticated nests, chemically mark the inside of their nest, and, differently, their nest entrances, nest surroundings and foraging area [17]. They generally use an alarm signal, a trail pheromone, and a recruitment signal [16]; they are able to navigate using memorized visual and olfactory cues [18 and references therein]; they efficiently recruit nestmates where, when and as long as it is necessary [19], and, finally, they clean their nest and provide their area with cemeteries [20]. According to the complexity of their society and their behavior, it looks reasonable to use ants as biological models for studying physiological and ethological effects of substances, treatments or situations.

During many years, we worked on ant species belonging to the genus *Myrmica*, and among others, on *Myrmica ruginodis* Nylander 1846. We know about its ecological traits, eye morphology [21], subtended angle of vision [22], visual perception [23], navigation system [24], visual and olfactory conditioning capabilities [25], and recruitment strategy [26]. The ontogenesis of cognitive abilities of *Myrmica* species has also been approached [27-32]. Studies on the impact of age, activity and diet on the conditioning capability of *M. ruginodis* [33] led to presume that ants could be good biological models. This was confirmed by the study of the effects of caffeine, theophylline, cocaine, and atropine [34], of nicotine [35], of morphine and quinine [36], of fluoxetine (an 'ISRS' antidepressant) [37], of anafranil (an 'ACT' antidepressant) and of efexor (an 'IRSNa' antidepressant) [38], of carbamazepine [39], and finally of buprenorphine and methadone [40]. Each time, we observed effects related to those observed on humans, and brought information and precision on them. Here, we used ants, and more precisely the species *M. ruginodis*, once more as a biological model for examining effects of a stevia/aspartame 91/9 0.123% aqueous solution.

Which Traits can we Effectively Correctly Examine?

The ants' food being given on their foraging area, at a clearly visible place, it is easy to assess food consumption. The ants' acquisition of a visual conditioning, as well as their visual memory can be quantified using an already set up experimental protocol. We are now accustomed to precisely assess the ants' locomotion (linear and angular speeds), precision of reaction (orientation towards an alarm signal), response to a pheromone (trail following behavior), audacity, brood caring, and cognition. The ants' preference between two kinds of liquid food is also easy to quantify using a well-tried analytical technique [40 + references therein].

Why have we no Conflict of Interest?

Doing fundamental research on the ethology of ants without external funding, we have no conflict of interest. On the contrary, we guess that several studies made on aspartame and stevia might have been done with some conflict of interest.

We are thus in the best situation possible for examining on ants the effects of a stevia/ aspartame 91/9 solution, all the more so since we have already studied the effects of each of these two sweeteners separately [6 and paper accepted for publication].

EXPERIMENTAL PLANNING

The ants of four colonies (see below) were first maintained under a sugar (saccharose) diet, and the ants' locomotion, orientation to an alarm signal, trail following behavior, audacity, brood caring behavior, and cognition were assessed.

The same ant colonies received then a diet of stevia/aspartame 91/9 and the same physiological and ethological traits were identically assessed (same protocols, same samples, same duration etc…).

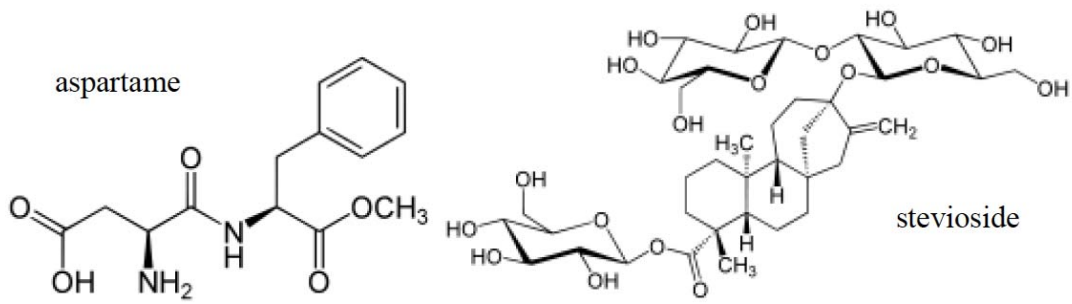

Figure 1: Chemical structure of the two studied sweeteners.

After that, the ants being still under the mixture of the sweeteners diet, we assessed their visual conditioning and memory.

Finally, we observed the ants' preference between sugar water and the here examined mixture, and thereafter their choice between sugar water and a stevia/aspartame 96/4 mixture.

MATERIAL AND METHODS

Collection and Maintenance of Ants

The experiments were performed on four colonies of the ant species *Myrmica ruginodis* Nylander 1846 collected in an old quarry of the Aise Valley (Ardenne, Belgium), on the borders of a forest, the ants nesting under stones or in wood. The colonies were demographically similar, each containing a queen, brood and about 500 workers. All the colonies were maintained in the laboratory in artificial nests made of one to three glass tubes half-filled with water, a cotton-plug separating the ants from the water. The humidity inside the artificial nests was thus similar to that usually existing inside natural nests. The glass tubes were deposited in trays (34 cm x 23 cm x 4 cm), which internal sides were slightly covered with talc to prevent the ants from escaping. These trays served as foraging areas, food being delivered in them. The ants were fed with a 30% saccharose aqueous solution (sugar water) provided *ad libitum* in a small glass tube plugged with cotton, and with two cut *Tenebrio molitor* (Linnaeus 1758) larvae provided twice a week on a glass slide. Such food corresponds to the natural requirements of the studied ant species. During experiments, the sugar water was replaced by an aqueous mixture of stevia + aspartame (see below) delivered to the ants exactly as their usual sugar water. Temperature was maintained between 18°C and 22°C with a relative humidity of circa 80% all over the course of the study. These are optimal conditions for the studied ant species. Lighting had a constant intensity of 330 lux while caring for the ants, training and testing them. During other time periods, lighting was dimmed to 110 lux. The ambient electromagnetic field had an intensity of 2-3 $\mu W/m^2$. All the members of a colony are here named nestmates, as commonly done by researchers on social hymenoptera.

Aqueous Solutions of Stevia/Aspartame 91/9 and 96/4 w/w

Stevia was furnished by the pharmacist J. Cardon (1050, Bruxelles) in the form of tablets containing 21 mg of stevia, made by the manufacturer 'Axone Pharma SA' (Braine L'Alleud, Belgium). Aspartame was furnished by the same pharmacist in the form of tablets (85 mg) containing 8.5 mg of aspartame, made by the manufacturer 'Canderel', one of the most available sources of aspartame and the most consumed one. According to the manufacturers, one tablet of either of these sweeteners must be used instead of one small spoon of sugar in order to have the same sweet power, i.e. one tablet into 150-180 ml liquid. However, the aqueous solution of the two sweeteners to be given to the ants must be equivalent in sweetness to the sugar water they usually consume in the wild and in laboratory, which is nearly saturated in glycosides (glucose, saccharose …). For instance, to feed them in laboratory, we pour ten small (coffee) spoons of sugar (= 5 gr x 10 = 50 gr) into 150 – 200 ml of tap water to obtain a sugared solution the ants obviously appreciate. For obtaining the same sugared taste using stevia or aspartame, ten tablets of one or the other sweetener must be dissolved into 150 – 200 ml of water. As insects consume proportionally about ten times less water than mammals, for feeding ants with a quantity of sweeteners proportionally similar to that consumed by humans, we should use, as a matter of course, a solution of ten tablets into 150 ml water. Therefore, we dissolved 4 tablets of stevia and one tablet of aspartame into 75 ml of water (the concentration in sweeteners of the solution being: 4 x 21 mg + 1 x 8.5 mg = 92.5 mg in 75,000 mg of water =

0.123%) and so obtained a solution of stevia/aspartame 90.8/9.2 ~ 91/9 w/w. Five ml of that solution were poured into the kind of small tubes usually used to provide sugar water to the ants. The tubes were plugged with cotton which was refreshed each day, while the entire solution was renewed every two days. Note that the glycosides of stevia are stable even in water while the aspartame molecule is not and hydrolyzes in about two days. It was checked each day if ants consumed the aqueous solution. The ants effectively consumed it, apparently just like they consumed their usual sugar water, a trait we quantified at the beginning of the experimental work. For obtaining a solution of stevia/aspartame 95.7/4.3 ~ 96/4 w/w, we dissolved 9 tablets of stevia and one tablet of aspartame into 150 ml of water (the concentration in sweeteners of the solution being: 9 x 21 mg + 1 x 8.5 mg = 197.5 mg in 150,000 mg of water = 0.132%).

Ants' Complete Food Consumption

For assessing the ants' food consumption under a sugar water diet or under a stevia/aspartame 91/9 diet, we counted during five consecutive days, twice each day, exactly at the same time o'clock and under identical conditions (giving food or not, t°, humidity, light) for each kind of liquid diet, the ants present on the sugar or the sweetened water and on the provided *T. molitor* larvae (Table **1**, daily counts). We then established the mean value per day (= mean of 4 x 2 = 8 counts for each kind of food; in total 5 mean values for each kind of food; Table **1**, daily means), as well as the mean of all the counts (N = 40) performed for each kind of food (Table **1**, total means). For each kind of food (i.e. liquid or meat), the series of five daily means were compared between the two kinds of sweet diet (i.e. sugar or stevia/aspartame 91/9) using the non parametric test of Wilcoxon [41].

Ants' Locomotion (Linear and Angular Speeds) and Ants' Precision of Reaction (Orientation Towards an Alarm Signal)

All the assessments were made on ants freely moving on their foraging area. For each assessment, the movement of ten ants of each colony (n = 4 x 10 = 40 ants) was analyzed. Ants' linear and angular speed was assessed without presenting any stimulus to the ants. Ants' orientation towards an alarm signal (which allowed examining the ants' precision of reaction) was assessed by presenting to the ants, on their foraging area, an isolated worker's head (Figure **2A**). Such a head, with widely opened mandibles, is a source of alarm pheromone identical to that of an alarmed worker, in terms of the dimensions of the emitting source (the mandibular glands' opening) and of the quantity of pheromone emitted [42].

Trajectories were manually recorded using a water-proof marker pen, on a glass slide horizontally placed 3 cm above the experimental tray area, where the tested individuals were moving. A metronome set at 1 second was used as a timer for assessing the total time of each trajectory. Each trajectory was recorded until the ant reached the stimulus or walked for about 6 cm. All the trajectories were then copied with a water-proof marker pen onto transparent polyvinyl sheets. These sheets could then be affixed to a PC monitor screen and remained in place due to their own static electricity charge. The trajectories were then analyzed using specifically designed software [43], each trajectory being entered in the software by clicking as many points as wanted with the mouse (for instance, 20 points in a trajectory length of 5 cm) and by entering then the location of the presented worker's head. After that, the total time of the trajectory was entered, and the software was asked to calculate three variables defined as follows:

The linear speed (V) of an animal is the length of its trajectory divided by the time spent moving along this trajectory. It was here measured in mm/s.

The angular speed (S) (i.e. the sinuosity) of an animal's trajectory is the sum of the angles, measured at each successive point of the trajectory, made by each segment 'point i to point i – 1' and the following segment 'point i to point i + 1', divided by the length of the trajectory. This variable was here measured in angular degrees/cm.

The orientation (O) of an animal towards a given source (here a small blank piece of paper used as a control or an ant's head) is the sum of the angles, measured at each successive point of the recorded trajectory, made by each segment 'point i of the trajectory – given source' and each segment 'point i – point i + 1', divided by the number of measured angles. This variable was here measured in angular degrees. When such a variable (O) equals 0°, the observed animal perfectly orients itself towards the given source; when it equals 180°, the animal fully avoids the source; when O is lower than 90°, the animal has a tendency to orient itself towards the source and when it is larger than 90°, the animal has a tendency to avoid the source.

Each distribution of 40 values of each variable was characterized by its median and quartiles (since being not Gaussian) and the distributions obtained for ants under a stevia/aspartame 91/9 diet were compared to those obtained for ants under a sugar diet using the non-parametric χ^2 test [41]. Two distributions were considered as statistically different when $P < 0.05$.

Ants' Trail following Behavior

The ants' response to their trail pheromone was assessed, on the four colonies, by quantifying the reaction of ten ants of each colony (n = 4 x 10 = 40), to examine the ants' general response to their pheromones. The trail pheromone of *Myrmica* ants is produced by the workers' poison gland. Ten of these glands were isolated in 0.5 ml (500µl) hexane and stored for 15 min at -25 °C. To perform one experiment, 0.05 ml (50µl) of the solution was deposited, using a metallic normograph pen, on a circle (R = 5 cm) pencil drawn on a piece of white paper and divided into 10 angular degrees arcs. One minute after being prepared, the piece of paper with the artificial trail was placed in the ants' foraging area. When an ant came into contact with the trail, its movement was observed (Figure **2C**). Its response was assessed by the number of arcs of 10 angular degrees it walked without departing from the trail, even if it turned back while walking on the trail. If an ant turned back when coming in front of the trail, its response was assessed as "zero arc walked"; when an ant crossed the trail without following it, its response equaled "one walked arc". Before testing the ants on a trail, they were observed on a "blank" circumference imbibed with 50µl of pure hexane, and the control numbers of walked arcs were so obtained (Table **2**, C = control, T = test). On experimental trails, *Myrmica* workers do not deposit their trail pheromone because they do so only after having found food or a new nest site. Each distribution of values was characterized by its median and quartiles (since being not Gaussian). The distribution of values obtained for ants under a stevia/aspartame 91/9 diet was compared to that obtained for ants under sugar diet using the non parametric χ^2 test [41].

Ants' Audacity

This trait was assessed on the four used colonies. A cylindrical tower built in strong white paper (Steinbach ®, height = 4 cm; diameter = 1.5 cm) was set on the ants' foraging area, and the ants present on it, at any place, were counted 10 times, in the course of 10 min. The mean and extremes of the obtained values were established (Table **2**, audacity) and the values obtained under the two kinds of diet were compared to one another using the non parametric Mann-Whitney U test [41].

Ants' Brood Caring

This trait was assessed on colonies B and C which contained numerous larvae. A few larvae were removed from the inside of the nest and deposited in front of the nest tube entrance. For each colony, five of them were carefully observed, as well as the ants' behavior in front of a larva (Figure **2B**). For each colony, the larvae among the five observed ones still remaining out of the nest after 5 seconds, 2, 4, 6, 8, and 10 minutes were counted, and the numbers recorded for each colony were added (Table **3**, brood caring). The results obtained for ants under a stevia/aspartame 91/9 diet were compared to those obtained for the same ants under a sugar diet using the non-parametric Wilcoxon test [41], the values of N, T and P being given in the results section.

Ants' Cognition

The assessment was made on ants of colonies A and D using an adequate experimental apparatus schematically presented in [35: Figure **3**]. This apparatus consisted in a small tray (15 cm x 7 cm x 4.5 cm) inside of which pieces of white extra strong paper (Steinbach ®, 12 cm x 4.5 cm) were inserted in order to create a way with twists and turns between a loggia too narrow for 15 ants at a time (the initial loggia) and a larger one (the free loggia) (Figure **2D**). Two experimental apparatus were built and used, each one, for one of the two colonies. Each time 15 ants were collected from their colony and set all together, at the same time, in the initial loggia of the apparatus, and those located in this loggia as well as in the free loggia were counted after 5 seconds, 2, 4, 6, 8 and 10 minutes. The numbers obtained for the two colonies were added (Table **3**, cognition). The total numbers obtained for ants under a stevia/ aspartame 91/9 diet were statistically compared to those obtained for the same ants under a sugar diet using the non-parametric Wilcoxon test [41], the values of N, T, and P being given in the results section.

Ants' Visual Conditioning and Memory

These traits were examined on the four experimental colonies.

Briefly, at a given time, a green hollow cube was set above the pieces of *T. molitor* larvae given as food, the ants of the four colonies undergoing so visual operant conditioning. Tests were then performed in the course of time, while the ants were expected to acquire conditioning then, after having removed the green hollow cube, while the ants were expected to partly lose their conditioning.

In detail, ants were collectively visually trained to a green hollow cube constructed of strong paper (Canson ®) according to the instructions given in [25] and set over the meat food which served as a reward. The color has been analyzed to determine its wavelengths reflection [44]. The ants could see the cube and easily enter it. Choosing the way with the green cube (see below) was considered as giving the 'correct' choice when ants were tested as explained below.

Ants were individually tested in a Y-shaped apparatus (Figure **2E**) constructed of strong white paper according to the instructions given in [25], and set in a small tray (30 cm x 15 cm x 4 cm), apart from the experimental colony's tray. Each colony had its own Y apparatus. The apparatus had its own bottom and its sides were slightly covered with talc to prevent the ants from escaping. In the Y-apparatus, the ants deposited no trail since they were not rewarded. However, they could utilize other chemical secretions as traces. As a precaution, the floor of each Y-apparatus was changed between tests. The Y-apparatus was provided with a green cube [25] in one or the other branch. Half of the tests were conducted with the cube in the left branch and the other half with the cube in the right branch of the Y maze, and this was randomly chosen. Control experiments had previously been made on never conditioned ants and on trained ants of colonies being under sugar water diet [25: Figure **4**]. This had to be done because, once an animal is conditioned to a given stimulus, it becomes no longer naïve for such an experiment. It was thus impossible to perform, on the same ants, conditioning first under a sugar diet, then under a stevia/aspartame 91/9 diet. The only solution was to use previous results obtained in the course of identical experiments made on similar colonies being under sugar diet [25].

To conduct a test on a colony, 10 workers - randomly chosen from the workers of that colony - were transferred one by one onto the area at the entrance of the Y-apparatus. Each transferred ant was observed until it turned either to the left or to the right in the Y-tube, and its choice was recorded. Only the first choice of the ant was recorded and this only when the ant was entirely under the cube, i.e. beyond a pencil drawn thin line indicating the entrance of a branch (Figure **2E**). Afterwards, the ant was removed and transferred into a polyacetate cup, in which the border was covered with talc, until 10 ants were so tested, this avoiding testing twice the same ant. All the tested ants were then placed back on their foraging area. For each test, the numbers of ants belonging to the four used colonies (n = 10 x 4 = 40 ants) which chose the "correct" way with the green cube, or went to the "wrong" empty branch of the Y were recorded. The percentage of correct responses for the tested ant population was so established (Table **4**). The results here obtained for ants under a stevia/aspartame 91/9 diet were compared to those previously obtained for ants under a sugar water diet [25], using the non-parametric Wilcoxon test [41]. The values of N, T, and P, according to the nomenclature given in the here above reference, are given in the results section.

Preference between a Sugar Solution and a Stevia/Aspartame 91/9 or 94/6 Solution

Fifteen ants of colony A, as well as of colony B, were transferred into a small tray (15 cm × 7 cm × 5 cm), the borders of which had been covered with talc to prevent escape, and in which two tubes (h = 2.5 cm, diam. = 0.5 cm) were laid, one containing a 30% saccharose aqueous solution, the other an aqueous solution of stevia/aspartame 91/9 (the same solution as that used in the course of the whole experimental work), each tube being plugged with cotton. In one of the trays, the tube containing the sweeteners was located on the right; in the other tray, it was located on the left (Figure **2F**). The ants drinking each kind of liquid food were counted 12 times in 15 min, the mean values being then established for each kind of liquid. They were statistically compared to the values expected if ants randomly went drinking each kind of liquid, using the non-parametric goodness of fit χ^2 test [41].

Exactly the same handling, quantification and statistical analysis were performed on colonies C and D using a stevia/aspartame 96/4 solution.

RESULTS

Food Consumption

Under sugar diet, the ants of the four used colonies were present on the sugar solution as well as on the meat food just as they were before the experimentation. Having often worked on *M. ruginodis,*

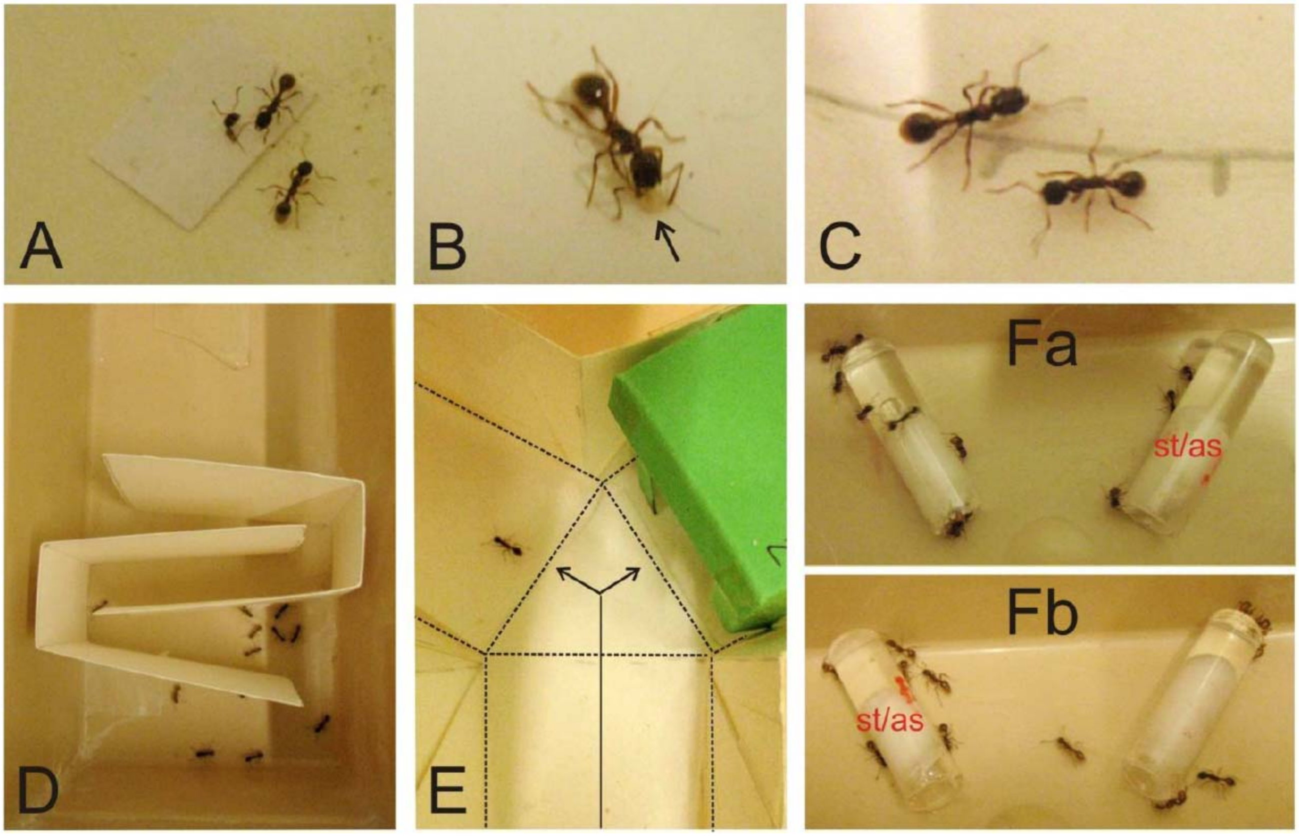

Figure 2: some views of the experiments. **A**: two ants reaching an isolated worker's head, a source of alarm pheromone. **B**: an ant 'gently' taking a larva between its mandibles. **C**: two ants following a circular trail. **D**: fifteen ants tested in an apparatus made of a small initial loggia (below, where they were firstly set), twists and turns, and a large loggia (above, were they were expected to go) for assessing their cognitive abilities. **E**: an ant, under visual conditioning, tested in a Y apparatus and giving the wrong response, i.e. moving into the branch not provided with a green cube (present on the right). **F**: ants of colonies A (**Fa**) and B (**Fb**) confronted to sugar water and a stevia/aspartame 91/9 solution (st/as written in red). Finally, they drank equally the two sweetened solutions.

we can state that the here observed ants' food consumption was perfectly normal i.e. the ants eat neither more nor less than they did usually. When the ants' diet was changed into a mixture stevia/aspartame 91/9, the ants' behavior obviously did not change: they went on foraging as usually and came onto the two kinds of food just as under a sugar solution diet (Table **1**). The daily means were statistically similar (ants on the sweetened liquid: N = 4, T = -8.5, P ~ 0.160; ants on the meat food: N = 4, T = 7, P = 0.313) although that, in average, under a stevia/aspartame 91/9 diet, the ants were slightly less numerous on the sweetened water (1.00 vs 1.12) and slightly more numerous on the meat food (0.80 vs 0.63), what allowed a slightly less sweeteners consumption. *In fine*, the total mean of the numbers of ants counted on the food was identical for the two kinds of diet: 0.90 *vs* 0.90. The total food consumption was thus not changed by a stevia/aspartame 91/9 diet, contrary to what occurred under either an aspartame or a stevia diet. We also checked the appearance of the ants' gaster: they were not exceptionally enlarged or not too little enlarged, but were just as those of ants under a usual diet (meat and a saccharose solution).

Linear and Angular Speeds

The ants' locomotion was not affected by a diet of stevia/aspartame 91/9. The numerical results (n = 40; Table **2**, table lines 1, 2) revealed that the ants' speed of locomotion was similar under a sugar and a stevia/aspartame 91/9 diet (12.8 mm/s *vs* 12.8 mm/s; χ^2 = 1.95, df = 2, NS) as was also their sinuosity of movement (136 ang. deg./cm *vs* 134 ang. deg/cm; χ^2 = 0, df = 1, NS).

Orientation Towards an Alarm Signal

While experimenting, we observed that ants under a stevia/aspartame 91/9 diet oriented themselves as well as when they were under a sugar diet, making correctly true positive taxis (Figure **2A**). The numerical results (n = 40; Table **2**, table line 3) indeed showed that the ants' orientation ability under a stevia/aspartame 91/9 diet was very similar to that presented under a sugar diet: 43.0 ang. deg. *vs* 44.8 ang. deg.; $\chi^2 = 1.89$, df = 2, NS. Thus, this solution of a mixture of stevia and aspartame did not impact the ants precision of reaction, i.e. a trait here pointed out *via* their orientation towards a source of alarm pheromone.

Trail Following Behavior

On the basis of our observation and the obtained numerical results (n = 40; Table **2**, table line 4), this trait, reflecting the ants response to their pheromones, was not affected when changing their sugar diet into a stevia/aspartame 91/9 diet. The ants went on correctly following the presented trail (Figure **2C**), and their mean distance walked along the trail was similar (9.0 *vs* 9.0 arcs of 10°; $\chi^2 = 2.91$, df = 3, NS). The ants also behaved as usually when encountering one another (Figure **2C**).

Audacity

Under sugar diet, the ants were not very inclined to climb on an unknown tower: meanly 1.1 ants were counted on the presented apparatus, and only one ant was seen moving on the tower. Under a stevia/aspartame 91/9 diet, the ants were also little inclined to move on the unknown apparatus and to climb on the tower. Meanly 1.0 ant was counted on the apparatus, and only one ant was seen climbing. The difference of ants' behavior under the two kinds of diet was not significant: U = 935.5, Z = 1.735, P = 0.083.

Table 1: Food consumption under a diet of 30% saccharose (sugar water) and a diet of stevia/aspartame 91/9 w/w, in aqueous solutions. Four colonies were provided with sugar water for five days, and two days later, with an aqueous solution of stevia/aspartame 91/9, as well as, each time with pieces of two *Tenebrio molitor* larvae given at days 1, 3, 5. For each diet, during five days, the ants present on the sugar or the sweetened water and on the meat food were twice counted (daily counts). Again for each diet, the mean of these eight counts was established for each kind of food (daily means), and finally the total mean of the counts was calculated for each kind of food and for all the food (total means). Experimental details and statistical results are given in the text. Briefly, ants consumed exactly the same amount of food under a sugar water diet and under a stevia/aspartame 91/9 diet

Diet:	**Sugar water**								**Stevia/aspartame 91/9 solution**							
Food:	**sugar water**				**meat**				**sweetened water**				**meat**			
Colonies:	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Daily counts																
Day 1	0	3	1	1	0	0	0	1	1	1	0	2	1	1	2	1
	0	3	0	2	0	1	0	1	1	0	1	1	0	1	1	2
Day 2	2	0	0	3	1	0	1	1	1	1	1	2	0	0	1	2
	2	2	1	1	0	0	1	1	1	1	0	1	0	0	1	1
Day 3	1	0	1	2	1	1	0	3	1	0	1	1	1	1	0	1
	0	1	0	2	1	0	0	3	2	0	1	2	0	1	1	1
Day 4	1	0	0	3	0	0	0	1	1	1	0	2	0	1	0	1
	1	1	2	1	1	0	1	1	1	0	0	2	0	2	0	0
Day 5	3	0	1	1	1	0	0	1	2	1	1	0	1	1	1	1
	3	0	1	1	2	0	0	0	2	2	0	2	2	1	1	0
Daily means																
Day 1	1.25				0.38				0.88				1.13			
Day 2	1.38				0.63				1.00				0.63			
Day 3	0.88				1.13				1.13				0.75			
Day 4	1.13				0.63				0.88				0.50			
Day 5	1.25				0.50				1.25				1.00			
Total means																
Days 1-5	1.12				0.63				1.00				0.80			
	0.90								0.90							

Table 2: Five ethological and physiological traits under a sugar water diet and a diet of stevia/aspartame 91/9 solution. The table gives the median (and quartiles) or the mean [and extreme] values of these traits. Details and statistical significance are given in the text. Briefly, none of these five traits were affected by the consumption of a solution of stevia/aspartame 91/9 instead of one of 30% saccharose. C = control (a blank circumference); T = test (with trail pheromone)

Diet → Traits ↓	sugar water	stevia/aspartame 91/9
Linear speed (mm/s)	12.8 (11.1 – 14.7)	12.8 (11.6 – 14.1)
angular speed (angular degrees/cm)	134 (117 – 152)	136 (126 – 145)
orientation (angular degrees)	44.8 (30.9 – 64.2)	43.0 (31.4 – 59.4)
trail following	C: 1.0 (1.0-1.0)	C: 1.0 (1.0 – 1.0)
(n° of walked arcs)	T: 9.0 (6.0 – 17.0)	T: 9.0 (6.0 – 14.0)
'audacity'(n° of ants)	1.10 [0 – 3]	1.00 [0 – 2]

Table 3: Two ethological traits under a sugar water diet and a diet of a stevia/aspartame 91/9 solution. The table gives the sums of the scores of two colonies. Explanation and statistical values are given in the text. Briefly, these two traits were not affected by a consumption of stevia/aspartame 91/9 instead of saccharose

Diet → Traits ↓		sugar water		stevia/aspartame 91/9	
Brood caring:					
n° of not re-entered larvae after:	5 sec	10		10	
	2 min	8		8	
	4 min	6		6	
	6 min	4		4	
	8 min	2		2	
	10 min	0		0	
Cognition:		in front	beyond	in front	beyond
n° of ants in front and beyond the twists and turns after:	5 sec	29	0	26	0
	2 min	23	0	21	0
in front = in a small initial loggia	4 min	18	0	20	0
	6 min	18	0	18	0
beyond = in a large free loggia	8 min	16	1	15	2
	10 min	15	3	13	2

Thus, a diet of stevia/aspartame 91/9 did not change the ants' audacity, i.e. their tendency in making rather risky acts.

Brood Caring Behavior

Under a sugar diet, as well as under a stevia/aspartame 91/9 diet, the ants took well care of their brood (Figure **2B**) and re-entered quickly larvae experimentally removed from the inside of the nest. The number of not re-entered larvae in the course of time among the ten ones observed was identical under the two kinds of diet experimentally used (Table **3**, brood caring). A diet made of stevia/aspartame 91/9 instead of saccharose thus did not impact the ants' brood caring behavior.

Cognition (Trait Requiring Brain Activity)

Under a sugar diet, the workers (of the presently tested species, *M. ruginodis*) left the initial small loggia of the experimental apparatus, and reached not quickly the large free loggia beyond twists and turns, in fact more slowly than did the workers of the ant species *M. sabuleti* [35-40]. When the sugar diet was replaced by a stevia/aspartame 91/9 diet, the *M. ruginodis* ants went on leaving the initial small loggia and reaching the large free one at the same speed, with the same 'efficiency' (Table **3**, cognition; Figure **2D**). The difference according to the ants' diet was not significant: initial loggia: N = 5, T = 12, P = 0.156; free loggia: N = 2, NS. It can thus be concluded that, for ants, drinking a stevia/aspartame 91/9 solution instead of a 30% saccharose solution did not affect their cognitive ability.

Visual Conditioning and Memory

These ethological abilities were not affected when consuming a stevia/aspartame 91/9 solution instead of a saccharose one (Table **4**).

Table 4: Visual conditioning and memory under either a diet of a stevia/aspartame 91/9 solution or a diet of 30% sugar water. Explanation and statistics are given in the text. Briefly, the mixture of sweeteners did not decrease the examined traits and even slightly enhanced them

Traits ↓	Diet → Colonies →	stevia/aspartame 91/9					sugar water
		A	B	C	D	%	%
Visual conditioning							
after:	7 hrs	7	6	6	5	60.0	47.0
	24hrs	7	7	6	6	65.0	60.0
	31hrs	7	6	7	6	65.0	63.3
	48hrs	8	7	8	5	70.0	65.0
	55hrs	7	8	8	6	72.5	75.0
	72hrs	8	7	8	9	80.0	81.7
Visual memory							
after:	7 hrs	8	8	7	8	77.5	70.0
	24hrs	8	6	7	6	67.5	65.0
	31hrs	6	9	6	6	62.5	62.0
	48hrs	5	7	7	5	60.0	50.0
	55hrs	8	5	6	6	60.0	62.0
	72hrs	7	6	6	6	62.5	60.0

Indeed, firstly, under the sweeteners diet, the ants acquired visual conditioning somewhat more quickly than under saccharose diet, presenting for instance a score of 65% instead of 60% after having been trained for 24 hours. Finally, after 72 hours, the ants reached a similar score, i.e. 80% instead of 81.7%. The difference of conditioning acquisition ability under the two kinds of diet was not significant: N = 6, T = 16.5, P = 0.132. Secondly, under the sweeteners diet, the ants more slowly lost their conditioning than when under saccharose diet, presenting for instance a score of 60% instead of 50% 48 hours after the removal of the visual cue. Finally, after 72 hours without the visual cue, they retained about 10% of their conditioning under each kind of diet. The difference of visual memory presented under the two kinds of diet was at the limit of significance, in favor of the sweeteners diet: N = 5, T = 14, P = 0.063.

When the acquisition of visual conditioning and the visual memory were considered all together, the difference between the two kinds of diet was significant: N = 11, T = 56.5, P = 0.018. Consequently, the mixture stevia/aspartame 91/9 did not decrease, and even slightly increased, the two examined ethological abilities.

Preference between a Solution of Saccharose and One of Stevia/Aspartame 91/9 w/w

When ants of colonies A and B were confronted to a solution stevia/aspartame 91/9 and a solution of saccharose, the two solutions having the same sweetness, they went drinking equally the two solutions (Figure **2F**). In the course of the 12 experimental minutes, 12 ants and 16 ones of colony A were counted on the sugar and the sweetened solutions respectively, while 9 ants and 14 ones of colony B were counted on the sweetened and the sugar solutions respectively. In total, 26 ants were thus seen drinking the sugar solution and 25 ones were seen drinking the sweetened solution. Such a result was not statistically different from that expected if ants should go randomly drinking each kind of solution: $\chi^2 = 2.91$, df = 1, NS. Ants thus accepted the presently examined solution of stevia/aspartame 91/9 just like they accepted their usual sugar water. This result is in agreement with that of the first experiment (Results section, Food consumption).

Preference between a Solution of Saccharose and One of Stevia/Aspartame 96/4 w/w

Aspartame having more adverse effects than stevia, we looked if ants would also accept a solution of stevia/aspartame 96/4 just as they do with a solution of saccharose. In front of these two sweetened solutions, ants of colonies C and D somewhat preferred the sugar solution. Indeed, in the course of the 12 experimental minutes, 21 ants and 9 ones of colony C were counted on the sugar water and the sweetened solution respectively, while 11 ants and 32 ones of colony D were counted on the sweetened solution and the sugar water respectively. In total, 53 ants were thus seen drinking the sugar solution and 20 ones the sweeteners solution, a result statistically different from that expected if ants should go randomly drinking each kind of solution: $\chi^2 = 14.92$, df = 1, P < 0.001. Ants were thus not inclined to drink a mixture of stevia/aspartame 96/4 and obviously preferred sugar water. Presuming that 4% of aspartame, the tastiest sweetener, was not enough for giving to stevia, which is a poorly tasty but safer sweetener, a nice sweet taste, we evaluated on ourselves the sweetness of the two kinds of mixture. Indeed, for humans, the stevia/aspartame 91/9 mixture tastes better (is more pleasant) than a 96/4 mixture, the latter being rather similar to pure stevia.

DISCUSSION

The two presently most used sweeteners are aspartame and stevia. They present adverse effects. Aspartame is not a true glycoside but it has a very nice sweet taste and gives to the brain the 'presence of sugar' information. Consequently, it impacts the individuals' ethology and physiology [6]. Moreover, it hydrolyzes into noxious substances [1, 2, 3]. Stevia is a true glycoside and consequently does not severely impacts behavior and physiology [paper accepted for publication]. But it has not a so nice sweet taste and so slightly influences the individuals' behavior. Moreover, it produces a small amount of steviol, a rather noxious compound [4, 5]. Being chemically entirely different, these two sweeteners have no chemical interactions (aspartame = two amino acids + a radical; stevia = glycoside), what is not the case, for instance, for aspartame and glutamate. But they appear to have nearly opposite physiological and ethological adverse effects. This incited us to examine which effects a mixture of the two sweeteners could have. Therefore, we studied on ants as biological models, as we did for aspartame and stevia, the ethological and physiological effects of the mixture stevia/ aspartame 91/9 w/w in aqueous solution and, unexpectedly, found that, indeed, such a mixture had no adverse effects, neither those of aspartame, nor those of stevia.

More precisely, the solution stevia/aspartame 91/9:

-) does not affect food consumption, while aspartame largely increases it and stevia somewhat decreases it,

-) does not affect the locomotion, the precision of reaction, and the response to pheromones while aspartame, but not stevia, largely impacts these traits,

-) does not affect the audacity while aspartame largely and stevia somewhat increase it,

-) does not affect brood caring behavior and cognition while aspartame largely and stevia very little impact these two traits,

-) does not affect visual conditioning acquisition and memory while aspartame drastically, and stevia very little, impact these abilities,

-) is accepted just like sugar water while aspartame is soon refused, and stevia progressively given up in favor of sugar water.

Even if these results lead to advocate the studied mixture as the best one, a solution of stevia/aspartame 96/4 was tested in a choice experiment. Such a mixture was unwanted in favor of sugar water. We thus concluded that a stevia/aspartame 91/9 0.123% solution constitutes an excellent, safe and tasty sweetener without or at least with very few adverse effects.

Our results can at least partly be explained by the facts that:

-) the amounts of stevia (91%) and of aspartame (9%) in the studied 0.123% solution are less than those present in a 100% stevia 0.140% solution or a 100% aspartame 0.0567% solution (note that 4/5 tablets of stevia at 0.140% + 1/5 tablet of aspartame at 0.0567% = 0.112% + 0.01134% = 0.12334%),

-) stevia is a true glycoside and consequently reduces the effects of aspartame resulting from the fact that the latter, though not being a glycoside, gives to the brain the false information that it is a sugar,

-) aspartame enhances the taste of stevia, reducing in this way the effects of stevia due to its poor sweet taste and consequently its limited consumption,

-) stevia somewhat reduces food consumption, decreasing so the demand of food due to aspartame. This limits the amount of ingested aspartame.

We have shown that an aqueous solution mixing 96% stevia and 4% aspartame is not appreciated. Even if the amount of aspartame would be less, such a mixture would have adverse effects because less consumed. We did not study a stevia/aspartame 70/30 or 80/20 mixture because of important adverse effects of aspartame. The here tested aqueous solution of stevia/aspartame 91/9 had no adverse effects and was accepted by ants just like sugar water. It can presently be estimated as the best possible mixture.

Experimentation using a stevia/aspartame ca 90/10 mixture in solution should now be undertaken on mammals (rats, mice, monkeys) for checking if no adverse effects could be detected. Tests, including the quantification of the intensity of sweetness, should also be made on humans (adults, children, diabetic persons). This should be really useful according to the large consumption of sweeteners, used as food additives, or incorporated in drinks, creams, cakes, and even pet food, all over the world. A safe, tasty, easily available sweetener is presently a need and the results of our current work suggest such a sweetener.

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